The blood-brain interface (BBI): a culture change

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Abstract

The blood-brain interface (BBI) is the subject of a new named series at *Brain, Behavior, and Immunity*. It is timely to reflect on a number of advances in the field within the last ten years, which may lead to an increased understanding of human behaviour and a wide range of psychiatric and neurological conditions. We cover discoveries made in solute and cell trafficking, endothelial cell and pericyte biology, extracellular matrix and emerging tools, especially those which will enable study of the human BBI. We now recognize the central role of the BBI in a number of immunopsychiatric syndromes, including sickness behaviour, delirium, septic encephalopathy, cognitive side effects of cytokine-based therapies and the frank psychosis observed in neuronal surface antibody syndromes. In addition, we find ourselves interrogating and modulating the brain across the BBI, during diagnostic investigation and treatment of brain disease. The past ten years of BBI research have been exciting but there is more to come.

The blood-brain interface (BBI) is the subject of a new named series in *Brain, Behavior, and Immunity*. In this inaugural issue of the series, we have found ourselves taking stock of ten years of research since we last reviewed this topic (Bechmann et al., 2007; Galea et al., 2007a). Progress has been made, but perhaps the most important change has been cultural, most notably in three areas.

Firstly, more caution is being exercised by the scientific community in the use of the word "barrier". A single word such as "barrier" can convey a very particular concept. The word "blood-brain interface", encouraged by this named series, is closer to the truth (Banks, 2016). The word "interface" is more permissive and implies less singularity, which is important to help conceptualize the facts that the interface is relative and regulated, not absolute, and occurs at several different surfaces.

Secondly, there is increasing acceptance that the BBI is multiregional. The capillary gliovascular unit in the brain parenchyma has been considered to be the main site of the BBI for many years. The brain interfaces with the blood in a variety of other ways: (1) interstitial fluid pathways along cerebral vessels, dural lymphatics, nasal lymphatics, cranial and spinal nerve roots to reach cervical and paraspinal lymph nodes, (2) the blood-cerebrospinal fluid interface at the choroid plexus epithelium, (3) the tanycyte barrier at the junction between circumventricular organs and brain parenchyma, (4) arachnoid granulations, and (5) bidirectional neural communication between the brain and the viscera. At the molecular level, there are ectoenzymes, receptors and transporters at these sites which regulate traffic into and out of the brain. Hence when studying cross-talk between the brain and the rest of the body, one needs to consider these pathways.

Finally, the other important cultural shift has been the increasing emphasis on human studies, using *in vivo* and *in vitro* techniques, since this is more relevant to human health and reduces, refines and replaces animal use in research.

Why is studying the BBI important? Bidirectional communication between brain and blood and vice-versa across the BBI determines how the organism responds to its external environment during social stress, or heat stress and dehydration and so forth, and internal environments for example during systemic or brain disease. In addition, we find ourselves interrogating and modulating the brain across the BBI, during diagnostic investigation and treatment of brain disease. It is timely to reflect on a number of advances in the field within the last ten years, which may lead to an increased understanding of human behavior and a wide range of psychiatric and neurological conditions. This

piece is not meant to be an exhaustive review, but more of an introduction to some recent highlights in BBI research. In such a short introduction, the contributions of many cannot be covered.

Glycocalyx

The glycocalyx is the outermost layer of the BBI, forming a gel-like polysaccharide layer lining the luminal surface of the brain endothelium. The glycocalyx has three main functions, acting as a molecular sieve, a reservoir for protective enzyme systems and a mechanotransducer (Reitsma et al., 2007). The glycocalyx has a negative charge which is frequently implicated in molecular sieving by repelling anionic proteins. However close observation of isoelectric focussing patterns of immunoglobulin G (IgG), whose pI ranges from 5.6 to 8.6 thereby crossing physiological pH, in healthy cerebrospinal fluid (CSF) and serum does not reveal a preponderance of positively charged IgG in CSF (Andersson et al., 1994). Recent data has clearly underlined the size selectivity of the glycocalyx, as the main contributor to its sieving function. Dextrans of increasing molecular weight are progressively excluded from the glycocalyx of systemic endothelium, such that 40kDa dextran is fully permeable while 70kDa and 500kDa dextrans are progressively less permeable (VanTeeffelen et al., 2013). Most cytokines and inflammatory mediators are smaller than 40kDa and would therefore be able to cross the healthy glycocalyx. Glycocalyx degradation would have implications for passage of larger plasma molecules such as IgG and complement. In systemic fenestrated endothelium, this would be sufficient to permit entry of these larger immune mediators into tissue, but breakdown of tight junctions (TJs) or increased transcytosis would be required at the BBI. At long last, the cerebrovascular glycocalyx has started to receive attention (Haeren et al., 2016). A recent study has suggested that the cerebrovascular glycocalyx may be specialized (Yoon et al., 2017). In their *in vivo* system, these investigators observed that cerebral venules had little or no glycocalyx (Yoon et al., 2017), unlike elsewhere, e.g. mesenteric and cremaster muscle venules (Yen et al., 2012). In addition the glycocalyx in cerebral capillaries and arterioles was not permeable to 70kDa dextran (Yoon et al., 2017), in contrast to the systemic glycocalyx (VanTeeffelen et al., 2013). Future studies will need to confirm whether cerebrovascular glycocalyx is more selective than its systemic counterpart. If there are differences in molecular composition of the cerebrovascular glycocalyx, these could be exploited to develop a circulating biomarker of glycocalyx damage specific to cerebral endothelium.

Endothelial cells

Cerebral endothelium is specialized in that it lacks fenestrations, has a continuous rim of TJs and exhibits a low rate of transcytosis. This constitutes a hindrance to the free passage of solutes and cells. Solutes cross this BBI via two routes: paracellular (in between cells, across TJs) and transcellular

(via vesicular activity). The permeability of substances is inversely proportional to their molecular size. The paracellular not transcytotic route determines this size-selectivity as shown in claudin-5 deficient mice (Nitta et al., 2003). Significant progress has been made in mapping the molecular control of these paracellular and transcytotic routes. Astrocytic foot processes, covering the abluminal surface of the endothelium, secrete factors such as sonic hedgehog (SHH) (Alvarez et al., 2011), angiotensin (Wosik et al., 2007) and apolipoprotein E (ApoE) (Bell et al., 2012) which control endothelial TJ protein expression. The SHH pathway has been found to maintain TJ integrity via endothelial receptor smoothened (Alvarez et al., 2011) and netrin-1 (Podjaski et al., 2015). Astrocyte-secreted ApoE3, but not ApoE4, acting through low density lipoprotein receptor-related protein 1, suppresses a permeability-inducing CypA–nuclear factor-κB–matrix-metalloproteinase-9 pathway in pericytes (Bell et al., 2012). Gut microbiota influence solute permeability by secreting small chain fatty acids which upregulate TJ expression (Braniste et al., 2014). Endothelial G-protein-coupled receptor (GPCR) Gpr124 is essential to protect BBI integrity via Wnt signalling during neuropathology such as ischemic stroke and glioblastoma (Chang et al., 2017). The transcytotic route has been shown to be regulated by major facilitator superfamily domain containing 2a (Mfsd2a), which suppresses caveolae-mediated transcytosis in CNS endothelial cells (Ben-Zvi et al., 2014) via regulation of CNS endothelial cell (EC) lipid composition (Andreone et al., 2017). Interestingly higher rates of transcytosis can be reversed with imatinib (Armulik et al., 2010), a tyrosine kinase inhibitor which is already in clinical use in the oncology setting.

ECs possess a number of pathogen-associated molecular pattern and cytokine receptors, so they can sense circulating danger signal and respond to it. EC activation is a process characterized by increased permeability, upregulation of cell adhesion, cytokine receptors and antigen presentation molecules on the cell surface, a prothrombotic phenotypic change, and cytokine production (Wu et al., 2017). The role of ECs at the BBI is usually studied in the immune-to-brain direction, typically in response to systemic inflammation (Ching et al., 2007; Gosselin and Rivest, 2008; Wohleb et al., 2014). ECs can also respond to the brain's internal environment and signal to the periphery, and therefore may participate in "inside-out" signalling. For instance, ECs display peptides on the luminal aspect to trigger CD8 T cell entry into the brain (Galea et al., 2007b) and they upregulate luminal VCAM-1 in response to cerebral ischemia (Quenault et al., 2017). BBI solute permeability in response to systemic inflammation was higher in the presence of brain disease (Elwood et al., 2017), suggesting that brain disease increases EC responsiveness to systemic inflammation.

Little attention has been paid to EC turnover at the BBI. The normal brain has the lowest EC endothelial turnover time in the body, roughly about 1000 days, compared to around 100 days in the systemic vasculature (Hobson and Denekamp, 1984). The reason for this difference is unknown but since EC proliferation is increased by a number of inflammatory stimuli (Alonso et al., 2005; Gotts and Chesselet, 2005; Hellsten et al., 2004) the low EC turnover rate in the brain may be linked to locally suppressed immune responses. Hyperpermeability precedes and accompanies EC proliferation (Nag, 2002), so inflammation either within the CNS (during neurological disease) or the blood compartment (during systemic inflammatory states), may lead to increased loss of BBI integrity as a result of EC proliferation. Chronic inflammation and increased EC turnover leads to EC replicative senescence, a cellular state defined by cell cycle arrest (Hornsby, 2010). Accumulation of senescent ECs is associated with disrupted TJs and increased permeability *in vitro* and *in vivo* (Yamazaki et al., 2016). Senescent cells typically have a senescence-associated secretory phenotype (SASP), during which production of pro-inflammatory molecules stimulate leukocyte migration, activation and infiltration (Lasry and Ben-Neriah, 2015). Hence there is potential for an injurious cycle between inflammation, EC proliferation and EC senescence which can spread beyond the initial focus. This remains to be explored in detail.

The endothelium is monitored by the body's immune system. Non-classical monocytes Ly-6C^{low} monocytes under control of the transcription factor Nr4a1, continuously patrol vascular endothelium in the steady state, especially within capillaries (Auffray et al., 2007), scavenging microparticles from their lumenal side and mediating their disposal when damaged (Carlin et al., 2013). Whether this system is operative in the brain capillary network is unclear. Circulating endothelial progenitor cells (EPCs) (Asahara et al., 1997) play an essential role in regenerating endothelium. They have been studied in the context of stroke where low blood EPC levels confer a worse prognosis and EPC infusion improves outcome in preclinical models of stroke (Liman and Endres, 2012). The study of EPC recruitment and local EC proliferation in relation to the maintenance of a healthy BBI after inflammatory challenge is likely to deliver novel insights.

Pericytes
Pericytes, previously a poorly characterized cell type, have been the focus of intense study in recent years. Advances in pericyte biology have recently been possible due to the availability of platelet-derived growth factor (PDGF) mutants (Armulik et al., 2010; Lindblom et al., 2003) and mice expressing specific markers under control of the neural/glial antigen 2 (NG2) promoter (Zhu et al., 2008) and the PDGF receptor β promoter (Cuttler et al., 2011). It is clear that pericytes are a

heterogenous population of cells (Attwell et al., 2016), and further progress in the field will be facilitated by the recent description of a unique marker (NeuroTrace 500/525) staining a subpopulation of pericytes which lack smooth muscle actin, do not contract, have slender longitudinal processes and are located distant from arterioles in the mid-capillary bed (Damisah et al., 2017). Pericytes have been shown to be key in regulating BBI solute permeability by influencing endothelial transcytotic rate and by guiding astrocytic foot processes to cerebral vessel walls and mediating their polarization (Armulik et al., 2010). Additionally proximal pericytes expressing smooth muscle actin have an important gate-keeping role at first order capillary branches (Hall et al., 2014), determining downstream blood flow in response to neuronal activity and injury. Regional blood flow is an important determinant of cross-talk across the BBI; in other words the BBI will be more permissive at higher regional cerebral blood flow.

Basement membrane

The cerebrovascular basement membrane between the endothelial and glial layer is a key component of the BBI. Basement membrane molecules are mainly composed of collagen IV, laminins, nidogens and heparan sulfate proteoglycans, secreted by endothelial cells, pericytes and astrocytes. Recent research has shown that basement membrane proteins are important in maintaining BBI integrity to solutes and cellular transfer. In mouse experimental autoimmune encephalomyelitis (EAE), patchy laminin α5 (laminin 511) expression in the basement membrane inhibits T lymphocyte migration mediated by the binding of integrin α6β1 to laminin α4 (laminin 411) (Wu et al., 2009). Astrocytic laminin α2 (laminin 211) is essential for maintaining BBI solute integrity by regulating pericyte differentiation towards a resting state via binding to the integrin α2 receptor on pericytes (Yao et al., 2014). Resting, not contractile, pericytes maintain BBI integrity by inducing polarization of astrocytic endfeet and expression of TJ proteins in endothelial cells (Yao et al., 2014).

Interstitial fluid drainage

The parenchymal extracellular space is in direct continuity with the capillary basement membrane of the BBI (Morris et al., 2016). Solutes injected into the parenchyma first appear in the capillary basement membranes and then in the tunica media of leptomeningeal arteries (Morris et al., 2016), after which they appear around the internal carotid artery and finally cervical lymph nodes (Szentistvanyi et al., 1984). Substances injected into the subarachnoid space can enter the cortical surface by advection with CSF through the peri-arterial glial-pial basement membrane (Iliff et al., 2012; Morris et al., 2016; Schain et al., 2017). Whether the proximity of these two opposing

pathways represents a dual carriageway (or divided highway for American readers) into and out of the brain is uncertain, and further study is needed. An alternative aquaporin-4 dependent drainage system called the glymphatic pathway has been put forward (Iliff et al., 2012). In this pathway, solutes move by advection, carried along by bulk flow or convection of interstitial fluid, from the peri-arterial glial-pial membrane, through the interstitial space, towards the perivenous space, and then into venous sinuses. However this hypothesis is not in agreement with results from subsequent multiple experiments with differing approaches (Arbel-Ornath et al., 2013; Holter et al., 2017; Jin et al., 2016; Morris et al., 2016; Smith et al., 2017). One issue affecting this area of research is the near-universal experimental technique of using acute injection to deliver tracer, in that the volumes delivered may open up non-physiological pathways e.g. 10µL intracisternal injection (Iliff et al., 2012) when the total CSF volume in mouse is 40µL (Rudick et al., 1982) and 0.5-1 µL intrastriatal injection (Morris et al., 2016) when interstitial spaces are about 60nm in diameter (Thorne and Nicholson, 2006). Novel approaches are needed to study interstitial fluid which do not disturb the status quo during the experiment, such as direct water imaging, transgenic expression of tracers and allogenic/xenogenic transplants. The other issue is how many of these new discoveries in rodents apply to the human brain. Research in this area is intense and important for researchers working on the BBI.

Cellular movement

In the healthy brain, it has become increasingly clear that very little, if any, leucocyte influx occurs into the brain parenchyma proper. A detailed cytometry study confirmed that infiltrating cells were mainly located in the meninges and choroid plexus, so that infiltrating cells constituted less than 2% of total brain immune cell population within the brain parenchyma, with the vast majority being resident microglia (Korin et al., 2017). This is likely to be the case in man, since lymphocytes and monocytes are rarely seen in the normal brain parenchyma during clinical neuropathology practice. In contrast to the parenchyma, leucocytes enter the healthy CSF from the choroid plexus (Kivisakk et al., 2003); this is a selective process dependent on endothelial P-selectin binding, and nearly excludes B cells from healthy CSF.

In neurodegenerative disease, activation of the innate immune system occurs. It has now been clarified that influx of blood-derived immune cells into the parenchyma during Alzheimer-like pathology is almost non-existent, or short-lived and non-consequential (Prinz and Priller, 2017), with previous observations being put down to the effects of brain irradiation for bone marrow transplant (Mildner et al., 2011).

During overt inflammatory disease characterized by activation of the adaptive immune system, lymphocytes enter via leptomeningeal vessels, choroid plexus and post-capillary venules. Leptomeningeal microvessels have an imperfect TJ system, lower electrical resistance and reduced endothelial barrier antigen expression (Allt and Lawrenson, 1997); this lower threshold is in keeping with the observation that the first wave of lymphocyte influx into the brain during EAE occurs from these vessels (Bartholomaus et al., 2009). A centripetal invasion of lymphocytes into the brain from the subarachnoid space, during which the CSF acts as a carrier and reservoir for T cells, has been proposed in rodents (Schlager et al., 2016), but this does not fit in with the distribution of multiple sclerosis lesions in man. Although surface grey matter demyelination occurs and this is associated with meningeal inflammatory cells (Howell et al., 2015), it is not clear what comes first, and lesions in the white matter are more frequent towards the ventricles. Hence caution needs to be exercised when extrapolating from rodent to man.

Regarding egress from the brain, intraparenchymal monocytes have been traced moving along white matter tracts towards the cribriform plate and hence into cervical lymph nodes (Kaminski et al., 2012). In mice, CSF lymphocytes enter dural lymphatics to reach deep cervical lymph nodes (Louveau et al., 2015), yet the significance of this route in man in uncertain, especially given the knowledge that cervical lymph node metastasis from intracranial tumours is rare (Mondin et al., 2010).

Tools to study the BBI

To enable human *in vivo* study of the BBI, non-invasive *in vivo* measurement of BBI permeability is important. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) using gadolinium chelates as contrast agents can measure physiological BBI permeability (Cramer and Larsson, 2014) to a level equivalent to that measured by positron emission tomography (Iannotti et al., 1987). However the absence of a change in permeability does not mean it is absent, since contrast agents in current clinical use may not be able to measure low levels of BBI permeability. Gadoflourine M, an experimental contrast agent with high affinity to extracellular matrix proteins (Meding et al., 2007), is able to measure BBI permeability changes undetectable using the similarly sized and charged gadolinium-DTPA (Bendszus et al., 2008; Stoll et al., 2009). Further advances in the field of DCE-MRI are likely to increase sensitivity. Emerging imaging techniques to study the BBI include chemical exchange saturation transfer (Wu et al., 2016) and hyperpolarized contrast (Roy et al., 2016). Ultrasmall superparamagnetic iron oxide (USPIO) nanoparticle neuroimaging of immune cells is possible in humans (Daldrup-Link, 2017). At the moment the technology lacks sensitivity to cell type

and origin (resident versus blood-derived) (Kirschbaum et al., 2016), but further progress is likely to deliver benefits.

Molecular imaging of cerebrovascular EC activation holds promise for BBI research. The last few years has seen a number of PET and MRI molecular probes, some of which have been used in the clinical setting, mostly in the field of atherosclerotic vessel wall imaging (Vrachimis et al., 2016). MRI offers a much higher resolution than PET and is radiation free, and therefore offers significant advantages. Conjugation of anti-P-selectin antibodies to USPIO has enabled *in vivo* molecular imaging of cerebrovacular EC activation in the context of ischemia (Quenault et al., 2017) and EAE (Fournier et al., 2017).

In vitro gliovascular BBI models have the advantage of delivering information while reducing the need for animal use, and if human cells are used, this increases clinical relevance. The construction of three-dimensional all-human BBI models incorporating ECs, pericytes, astrocytes and basement membranes proteins, with real-time monitoring of TEER and capacitance (Maherally et al., 2017) is a technical achievement which will push BBI research into new territory. Human induced pluripotent stem cells have been used to derive endothelial cells, astrocytes and neurons from the same patient and recapitulate that individual's gliovascular BBI *in vitro*, with a TEER and permeability close to that observed *in vivo* (Canfield et al., 2017). Current efforts aim to deliver models closer to the real thing, incorporating shear stress and hypoxia.

The future

BBI research over the last ten years has been exciting and there is more to come. We now recognize the central role of the BBI in a number of recently recognized immunopsychiatric syndromes (Khandaker and Dantzer, 2016), including sickness behaviour (Kelley et al., 2003), delirium (Cunningham and Maclullich, 2013), septic encephalopathy (Sonneville et al., 2013), cognitive side effects of cytokine-based therapies (Myint et al., 2009) and the frank psychosis observed in neuronal surface antibody syndromes (Zuliani et al., 2012), to mention a few. We are learning how to deliver therapeutic drugs across the BBI (Banks, 2016). Clearly we need to know the BBI "inside-out".

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